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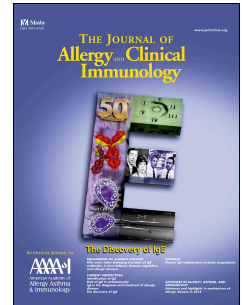
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Human Group 2 innate lymphoid cells do not express the IL-5 receptor

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Capsule summary: Group 2 innate lymphoid cells (ILC2s) are upstream regulators of IL-5-dependent eosinophil function in asthma. We've shown that ILC2s do not express the IL-5R and thus IL-5R-dependent therapeutic interventions (e.g. Benralizumab) are unlikely to be mediated directly on ILC2s.

Key words:

IL-5R α

Basophils

Eosinophils

Group 2 innate lymphoid cells

Benralizumab

Abbreviations:

IL-5 - Interleukin-5

IL-5R α – Interleukin-5 receptor α -subunit

ILC2s – Group 2 innate lymphoid cells

GMFI – geometric mean fluorescence intensity

SED – Super enhanced Dmax

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To the Editor,

Eosinophils, cardinal effector cells of type-2 inflammation, contribute to the clinical and immunopathological manifestations of asthma¹ and COPD² inflammatory endotypes. Eosinophil biology is governed by Interleukin (IL)-5, a cytokine that binds with high affinity to a specific IL-5 receptor α -subunit (IL-5R α) prior to forming a heterodimeric receptor complex with the β - subunit (β c)³. IL-5 signalling promotes differentiation, maturation and survival of eosinophil committed progenitors whilst acting on mature eosinophils to enhance their migratory potential and effector responses⁴. IL-5 signalling also promotes alternative splicing of the IL-5R α gene to generate transmembrane forms of IL-5R α ⁵. Since the IL-5-IL-5R axis appears to be restricted to eosinophils and basophils (and their progenitors)⁶, therapeutic regulation of these cells through the neutralisation of circulating IL-5 (e.g. mepolizumab and reslizumab)¹ or IL-5R α ligation (e.g. Benralizumab)^{1, 2}, have emerged as effective strategies to deplete blood, tissue and airway eosinophils and consequently reduce exacerbation rates and improve lung function¹.

Basophils and Group 2 innate lymphoid cells (ILC2s) are important innate sources of type-2 cytokines, including IL-5, in response to epithelial derived cytokines such as IL-33^{7, 8}. Group 2 innate lymphoid cells (ILC2s), however, have emerged as central and critical innate co-ordinators of steady state eosinophilopoiesis and epithelial cell driven, type 2 immunopathology in asthma⁸. As an important upstream regulator of eosinophil function we asked whether ILC2s, like their basophil counterparts, express the IL-5R α sub-unit since this may have important implications for our understanding of the role of the IL-5-IL-5R axis in disease and therapeutic targeting of these rare but important innate immune cells. Detailed methods are provided in the online repository.

ILC2s are rare innate lymphocytes that lack the T cell receptor complex and all known lineage markers but, similar to other type 2 cytokine producing cells such as Th₂ cells, eosinophils and basophils, express the type 2 prostaglandin D₂ receptor, DP2/CRTh2 (CD294)⁹. We defined blood ILC2s as cells with singlet, lymphocyte light scatter properties (Fig 1A-B), lineage (CD2, 3, 14, 16,

19, 56 and 235a)⁺ but CD294⁺ (Fig 1C). A large proportion of cells within this gate were basophils, (CD123⁺ cells in Fig 1D), however, CD294⁺, CD123⁺ ILC2s were present (Fig 1D and light scatter in E). Basophils displayed a higher level of CD125-PE staining than the ILC2s (Fig 1D) that could be blocked in the presence of rhIL-5 (Fig 1F), confirming the specificity of the antibody and basophil IL-5R α expression. In contrast to basophils, there was no change in CD125-PE signal intensity when ILC2s were incubated with rhIL-5 indicating that there was no IL-5R α expression on these cells (Fig 1F). To confirm the reproducibility of these findings, we recruited 6 control and 13 volunteers with asthma in each group (clinical details in Table E1 of the online repository) and measured IL-5R α expression. The total mean (SD) % of ILC2s within the lymphocyte gate for both control and asthma groups were 0.1 \pm 0.1% and 0.03 \pm 0.03%, respectively ($p=0.06$, Mann Whitney). In total, combining asthma and control data sets, the basophil CD125 geometric mean fluorescence intensity (GMFI) (mean \pm SD) in the absence or presence of rhIL-5 was 1285 \pm 614 and 401 \pm 315 respectively, representing a significant reduction in GMFI (Fig 1G and I). In contrast, the ILC2 CD125 GMFI (mean \pm SD) in the absence or presence of rhIL-5 were 45 \pm 45 and 42 \pm 33, respectively (Fig 1H and I). In a subset of samples ($n=3$), where basophils and ILC2s could be detected in whole blood, data were qualitatively similar. There were no significant differences between the control and asthma subjects for any of the flow cytometric measurements obtained.

We next sought to confirm whether the IL-5R α data was reflected at the RNA level using real-time PCR probes spanning the exon boundaries present within IL-5R α subunit variants (Fig E1A). For this, ILC2s were isolated from 3 additional donors and cultured in the absence or presence of cytokines known to enhance survival (IL-2 and IL-7) and activate ILC2s (IL-25 and IL-33). RNA was isolated at 6 time points (Day 0, 1, 2, 4, 7 and 14) and converted into cDNA. Eosinophil cDNA was included as a positive control. The IL-5R α transcripts were not detected in ILC2s at any time point, however, an increase in IL-5 mRNA was observed indicating that the culture conditions were sufficient to activate the cells (Fig E1 B and C).

Finally, using a similar approach to the blood analyses (Fig 1) and utilising surgically removed lung tissue from 7 subjects (Table E2) we asked whether tissue-derived ILC2s and basophils expressed IL-5R α . Tissue-derived basophils were defined as viable, CD45⁺Lin⁻CD294⁺CD123⁺CD127⁻ cells and ILC2s as viable, CD45⁺Lin⁻CD294⁺CD123⁻CD127⁺ cells present within the singlet lymphocyte gate (Fig 2A-E). Basophil CD125-PE GMFI (612 \pm 327 mean \pm SD) could be reversed in the presence of rhIL-5 to a mean(\pm SD) of 317 \pm 91 (example in Fig 2F and cumulative data in 2H). Using the super enhanced Dmax (SED) algorithm to compare basophil populations stained in the absence or presence of IL-5 for each of the 7 donors, revealed that 71.1 \pm 4.2% (mean \pm SD) basophils were CD125 positive (Fig 2I). In contrast, ILC2 CD125 GMFI (mean \pm SD) was significantly lower than basophils and showed little change in the presence of rhIL-5 (190 \pm 42 and 145 \pm 49, respectively) (example in Fig 2G and cumulative data in 2H). SED analysis of ILC2s stained in the absence or presence of rhIL-5 for each of the 7 donors (Fig2I) did not reveal a CD125 positive subset (mean \pm SD 3.3 \pm 0.89%). Data from these 7 subjects consistently show that tissue-derived basophils express the IL-5R α whereas tissue-derived ILC2s do not (Fig 2H).

The strength(s) of this study lies in the fact that we have measured protein IL-5R α subunit expression in a rare critical regulator of type 2 inflammation, ILC2s, and in the context of rhIL-5 mediated receptor blockade/downregulation, rather than an isotype control which is less robust. The robustness of our data using human peripheral blood (from asthmatic donors), *ex vivo* activated cells and lung tissue cells diminishes the likelihood that IL-5R α expressing ILC2s are present in asthmatic tissue. Although it would be desirable to extend our blood and tissue observations to investigate whether ILC2s in bronchial biopsies from asthmatics express IL-5R α the paucity of tissue ILCs and the need for multiple immunological markers to positively identify them severely limits this approach. These results extend the list of cells⁶ that are known not to express the IL-5R α subunit, specific for the biological activities of IL-5. Moreover, we show that rare tissue derived basophils, like their blood counterparts, express the IL-5R α . Our data suggest that the success of therapeutic interventions targeting IL-5/R are unlikely to be mediated directly on ILC2s but may function via both eosinophils and basophils.

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146
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150

151 **Figure 1** CD125 protein is absent on blood ILC2s. **A**, total PBMCs, singlets within boxed region. **B**,
152 singlet light scatter with lymphocyte gate overlaid. **C**, lineage⁻, CD294⁺ cells were identified (boxed
153 region) encompassing **D**, CD123⁺ basophils and CD123⁻ ILC2s, in the upper and lower left quadrant,
154 respectively with GMFI values. **E**, ILC2 light scatter properties are shown. **F**, Overlay of basophil and
155 ILC2 CD125 stained cells in the absence (red) or presence (blue) of rhIL-5. **G**, basophil and **H**, ILC2
156 CD125 GMFI paired data (mean±SD) from 6 healthy (open) and 13 asthma (filled) volunteers, also
157 shown in **I**, unpaired on the same axes. Statistical comparisons were made using a Wilcoxon
158 matched-pairs signed rank test.

159

160 **Figure 2** CD125 protein is absent on lung derived ILC2s. **A**, total lung tissue cells, singlets within
161 boxed region. **B**, singlet light scatter properties with lymphocyte gate overlaid. **C**, viable CD45⁺ cells
162 (boxed region). **D**, shows lineage⁻ CD294⁺ cells (boxed region). **E**, ILC2s (CD123⁻CD127⁺) and
163 basophils (CD123⁺CD127⁻) were identified. **F**, depicts basophil CD125 fluorescence and **G**, ILC2
164 CD125 fluorescence in the absence (red) or presence of rhIL-5 (blue). **H**, Collated basophil and ILC2
165 CD125 GMFI (mean±SD) data ($n=7$). **I**, Proportion of CD125⁺ basophils and ILC2s (calculated using
166 SED), exemplar values given in F and G. Statistical comparisons were made using a Wilcoxon
167 matched-pairs signed rank test.

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